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Increased ultrasonic vocalizations and risk-taking in rat pups of sleep-deprived dams

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HIGHLIGHTS

- Increased crying in pups born to dams on acute sleep deprivation during pregnancy
- Sleep loss during pregnancy increases risk-taking behavior in pre-adolescent pups.
- USVs during ontogeny provide early signals to understand mother–child bonding.
- Maternal sleep during pregnancy influences the emotional development of babies.

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ABSTRACT

Ultrasonic vocalizations (USVs) in rodent pups are analogous to cries in human babies. There is reduction in USVs in pups after experimental deprivation of rapid eye movement sleep of dams during pregnancy. However, the effects of total sleep deprivation on the USVs of newborns and their emotional development are not documented. Male pups born to the rats that underwent total sleep deprivation for 5 h during the third trimester made higher vocalizations, when tested on early postnatal days (pnds) in an isolation-paradigm. Their anxiety-related behaviors during pnds 25–28, were tested using elevated plus maze (EPM). In comparison to the control pups, weanlings of sleep-deprived dams made increased entries into the open arms and higher mobility in the EPM. Enhanced distress calls during early pnds and reduction in risk assessment in weanlings indicate a link between the two behaviors. The USVs during ontogeny may provide early signals about altered emotional development.

1. Introduction

Cries of babies, immediately after birth and during early postnatal period, remain an enigma both in terms of cause and nature. This expression of distress by neonates is now recognized as a valid component in the developmental milestone of babies [1,2]. Neonates utilize the cry as a principal means to express their pervasive discomfort to isolation-induced stress [1,3]. Ultrasonic vocalizations (USVs) in rodent pups, which are analogous to cries in human babies, possibly signal stress/anxiety for an imperative retrieval by the dams [4–6]. It is also considered as a reflection of their anxiety traits or affective states [7–9]. Though recent reports indicated that decreased vocalization in neonates is associated with despair and desolation during early development [10–12], the significance of the increased USVs needs further investigation.

Maternal stress during pregnancy is emerging as a major concern due to increased reports of anxiety disorders and cognitive deficits in the offspring [13–16]. There are altered emotion and decline in cognition, vigilance, attention, memory, and risk-taking behavior after sleep restriction in women [17–21]. Sleep loss is a modern life style dependent stress across all age-groups [22]. Recently, it was demonstrated that a reduction in rapid eye movement (REM) sleep during the last trimester of pregnancy in rats adversely affected the rate and quality of vocalization in their pups [12]. Since sleep consists of REM and non-REM (NREM) components that are regulated by different mechanisms in the brain [23,24], it is likely that the total sleep loss might have different effects on the early development and vocalizations. There are practical difficulties and ethical issues, on studying the probable association between total sleep loss in pregnant women and its consequences on the newborn. To address this issue, the present study was designed in the rodent model to investigate the effects of acute maternal total sleep deprivation (TSD) of 5 h (TSDX5h) during the third trimester, on the USV profiles of pups from birth to weaning, using an isolation paradigm. This was followed by assessing their anxiety in the early adolescence in the elevated plus maze (EPM) test.

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2. Materials and methods

2.1. Subjects

The study was carried out on rat pups born to mothers that were sleep-deprived during the third trimester. Prior to mating, adult nulliparous female Wistar rats (body weight 220–240 g), were implanted with EEG and EMG electrodes under anesthesia for monitoring sleep onset [25]. Their sleep onset was assessed on the basis of electrophysiological parameters. The electrodes were soldered to an IC socket and covered with dental cement over the head as described elsewhere [25]. After a post-operative recovery period of 10 days, these females were kept with males of similar age for mating. Every day they were examined for vaginal plug formation in the morning. After confirmation of pregnancy, females were housed individually in polystyrene cages at controlled temperature (26 ± 1 °C) and light–dark schedule of 12 h (lights on at 06:00 h). Food and water were provided ad libitum throughout the experiment.

The pregnant rats (n = 10) were randomly distributed into two groups. In the first group of rats (n = 5) TSD for 5 h (9 am to 2 pm) was achieved by gentle manual handling, starting from gestational day 14 until day 19. They were sleep-deprived during their third trimester of pregnancy by gentle manual handling [26]. Electrophysiological signals from the pregnant rats, during 5 h of study, were monitored in a BIOPAC system (MP 150 system) using connecting leads [27]. After completion of 5 h sleep deprivation protocol, the recording leads were disconnected and the rats were left undisturbed in their home cages. These rats were monitored till their parturition in their home cages. The second group which did not undergo sleep deprivation was taken as control and monitored till their parturition. On day 1 of parturition, the litter size was brought down to 6 by culling the remaining pups in both the groups to maintain uniformity in maternal care. Mothers and pups were only very minimally disturbed during the experiment.

2.2. Ultrasonic vocalization measurement procedure

Male pups of control group (n = 13) and experimental group (n = 27), obtained from ten pregnant rats, were monitored for their USVs. Their USVs were recorded on brief isolation from their mothers, as described previously [12]. The control group pups provided data about the natural course of development of vocalizations on different postnatal days. These were compared with USVs of pups of TSDX5h group mothers. The USVs generated by individual pups on isolation were recorded on various pnds 1, 5, 9, 11, 15, and 21 for a period of 2 min at room temperature of 28 ± 1 °C. The home cage with the dam and the pups was carried to the testing room. Pups were placed inside a glass beaker (without any bedding), kept inside a sound attenuated chamber, to record USVs. The USVs were recorded using a microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) placed 10 cm above the pups. The microphone was connected to a pre-amplifier (Avisoft UltrasoundGate 416H, Avisoft Bioacoustics) and digitized sonograms were stored in a computer. The ambient temperature within the glass beaker, during the USV recording, was monitored using FLUKE True-rms digital multimeter (model 287/289; USA) with K type thermocouple containing chromel–alumel probe. It took 8 to 10 s to isolate the pups, one at a time, from the mother to a glass beaker. It was ensured that the USVs produced by the isolated pups did not reach the home cage, kept 2 m away, where the other pups remained with the mother. After USV recordings, body weights of the pups were taken.

2.3. USV signal analysis

The USVs were analyzed quantitatively and qualitatively using Avisoft SASLab Pro software (version 5.1). The Fast Fourier Transform (FFT) was done to generate the spectrogram (FFT length 256 points, frame size of 100% in flat top window with temporal resolution of 75%). The spectrogram was produced at frequency resolution of 977 Hz and a time resolution of 0.25 ms. On each test day, the parameters analyzed included (1) calling rates i.e. number of calls/min, (2) call types, (3) duration of calls, (4) total time spent in calling, (5) carrier or fundamental (F_0) and peak frequency of calls, (6) loudness and (7) temporal profile in call numbers (distribution of calls during first and second minute).

2.4. Anxiety testing using elevated plus maze

The weanlings were tested for anxiety on pnds 25–28 after weaning (early adolescence) in the EPM using ANY-maze video-tracking system (version 4.82) from Stoelting Co. (USA). The plexiglass EPM having two open arms (50 × 10 cm) and two closed arms (50 × 10 × 50 cm), arranged in plus shape, was kept at a height of 45 cm. The arms of same types faced each other and were connected through an open central zone (10 × 10 cm). At the beginning of the experiment, weanlings were placed in the central zone, facing one of the closed arms and test was conducted for 5 min. The parameters taken were time spent in the open and closed arms and the central zone, total distance traveled in 5 min, total distance traveled in different zones, number of line crossings in each zone, total mobile time and ethologically derived measures like grooming, rearing and head dipping (number of presses).

2.5. Statistics

One way analysis of variance (ANOVA) with repeated measures, and post-hoc comparison with Bonferroni correction were done to compare the natural developmental profiles of the USV number (calling rate) and duration (of call, F_0) in the control and TSDX5h groups over different postnatal days. Non-parametric analysis (Mann Whitney-U test) and Student's *t* test were used to analyze differences in parameters between two groups. The level of significance was set at $p < 0.05$ for all comparisons. Chi square test was performed to find the changes in call type distribution between the groups over the postnatal days.

2.6. Ethics

The study was approved and performed in accordance with the guidelines laid down by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

3. Results

Gentle handling of the pregnant rats in the experimental group, at the onset of their sleep resulted in a reduction of sleep by 97.41 ± 0.01% as compared to the control group of rats.

3.1. Quantitative changes in the USVs in pups during pnds

A total of 26,927 ultrasonic calls were obtained from the pups on various days. The total numbers of USVs per min were averaged for each developmental day studied in male pups (n = 13 in control and 27 in experimental group) obtained from 10 litters. The intra and inter group multiple comparisons were made for all days for pups in control and TSDX5h groups. In control pups, the calling rates were low during initial pnds 1–5, but significantly increased on pnd 9 and they reached the peak calling on day 11 (Fig. 1; one way ANOVA, $F_{5,67} = 29.55$, $p < 0.0001$). Thereafter, the calling rate was reduced, reaching to negligible values on day 21. In pups of TSDX5h group, similar developmental increases in vocalization were observed from pnds 1–9 (one way ANOVA, $F_{5,108} = 14.3$, $p < 0.0001$), but calling did not increase further on pnd 11.

In comparison to control pups, calling rate in the pups born to TSDX5h mothers were significantly higher during initial pnds 1–9 (Fig. 1). Thereafter, USVs made by pups born to TSDX5h mothers were

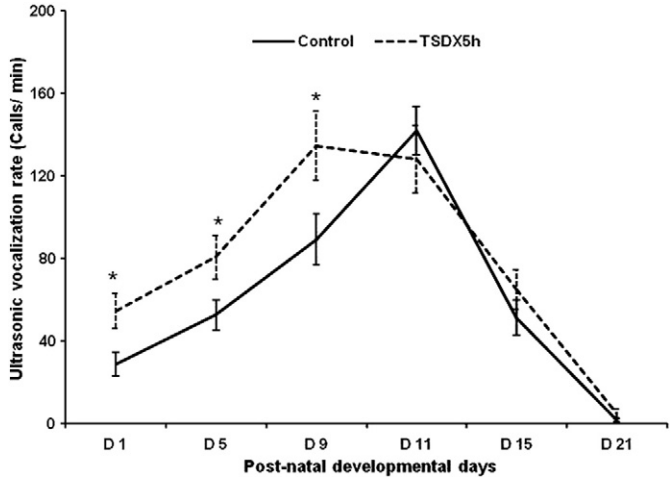

Fig. 1. Average no of USVs/min in pups after maternal separation. Vocalizations are presented as averaged calls/min ± SEM (standard error of mean) in the Y axis and different developmental days on X axis. 'D' refers to days, control values are represented in solid line while the TSDX5h group values are shown in dashed line. * designates $p < 0.05$ for comparison of values between control group v/s TSDX5h group.

comparable to the control pups without further peaking on day 11 (Fig. 1).

3.2. Call types on different days in control and TSDX5h group pups

Individual calls were labeled on the basis of shape and sonographic features, primarily in accordance with the prevailing classification [6,12,28]. USVs were grouped into five categories on the basis of number of syllables, frequency modulation and duration (Fig. 2). The constant frequency (CF) category consisted of flat calls, modulated frequency (MF) category in single syllable (upward, downward sweep, inverted U-shaped calls), two or three syllable calls, complex frequency modulation types (wave and complex), short calls and harmonics (Fig. 2). The percentages of calls in harmonics were calculated from the total calls per day, irrespective of the types that they belong to.

3.3. Distribution of call types on different days in control and TSDX5h group pups

The distribution of call types taken during the first minute of recording on different pnds are shown in Fig. 3. On pnd 1, flat type calls were predominant in both control and experimental group pups but significant variations were observed between different call types. The pups in TSDX5h group made higher number of two or three syllable calls and lesser number of flat calls, in comparison to the controls. No significant differences were observed on any other day in the distribution of call types (Fig. 3).

3.4. Changes in call durations (total and averaged)

Variations in the mean duration of different call types between the control and the TSDX5h groups are tabulated for various developmental days (Table 1). On pnd 1, upward calls (category Bi) in the TSDX5h group were significantly shorter in comparison to the control group ones. The 2-syllable calls were longer in the experimental pups on pnd 5. Towards pnd 15, flat and upward call durations were lower than in the control pups. The excess time spent in calling, by the pups of TSDX5h mothers on pnds 1–9, is evidently seen in Fig. 4.

3.5. Fundamental, peak frequency and bandwidth of calls

The carrier/fundamental frequency in the flat calls in the control and TSDX5h group pups remained around 40 to 43 kHz on pnds 1 and 5, increasing to 45–48 kHz on pnds 9–11, and reaching to a still higher frequency of 55–60 kHz on later days. The frequency overtones/harmonics in USVs were also common in both the groups. The peak frequency of calls in 2 syllables on pnds 1 and 5, and in the flat and wave types on pnd 15 were significantly higher in the TSDX5h group pups. On pnd 1, bandwidth in upward calls was significantly higher in the pups of the

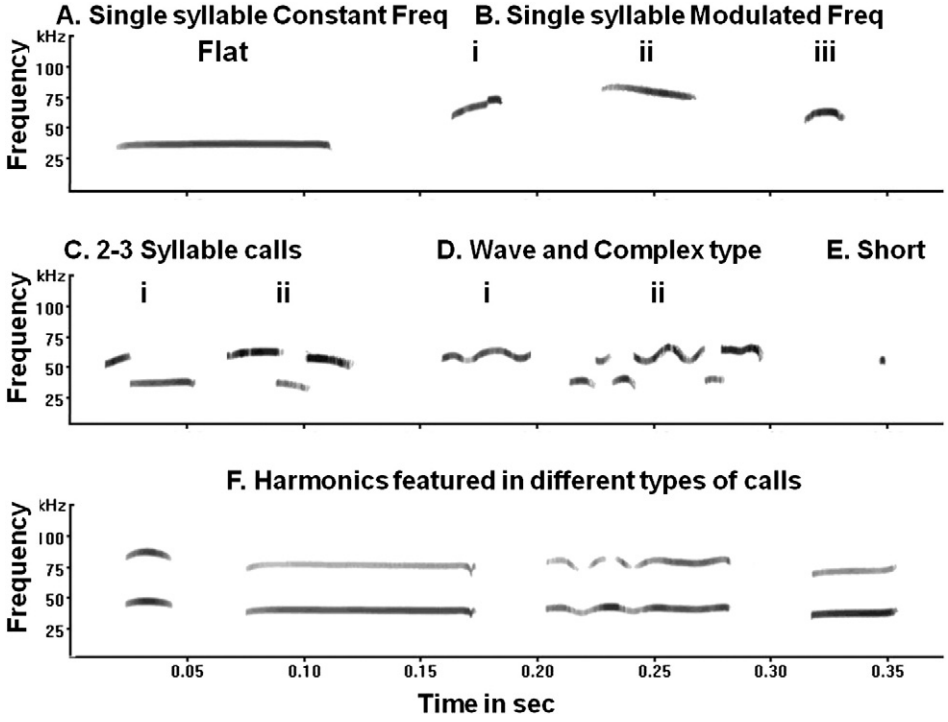

Fig. 2. Sonograph of different call types produced by pups in the isolation paradigm. The frequency is shown in kHz on the Y axis and time scale is expressed in sec on the X axis. In single syllable category (B) i, ii and iii denotes upward, downward and inverted U-shaped calls. In C and D, the numerals i, ii denotes 2 and 3 syllable, and wave and complex call types respectively.

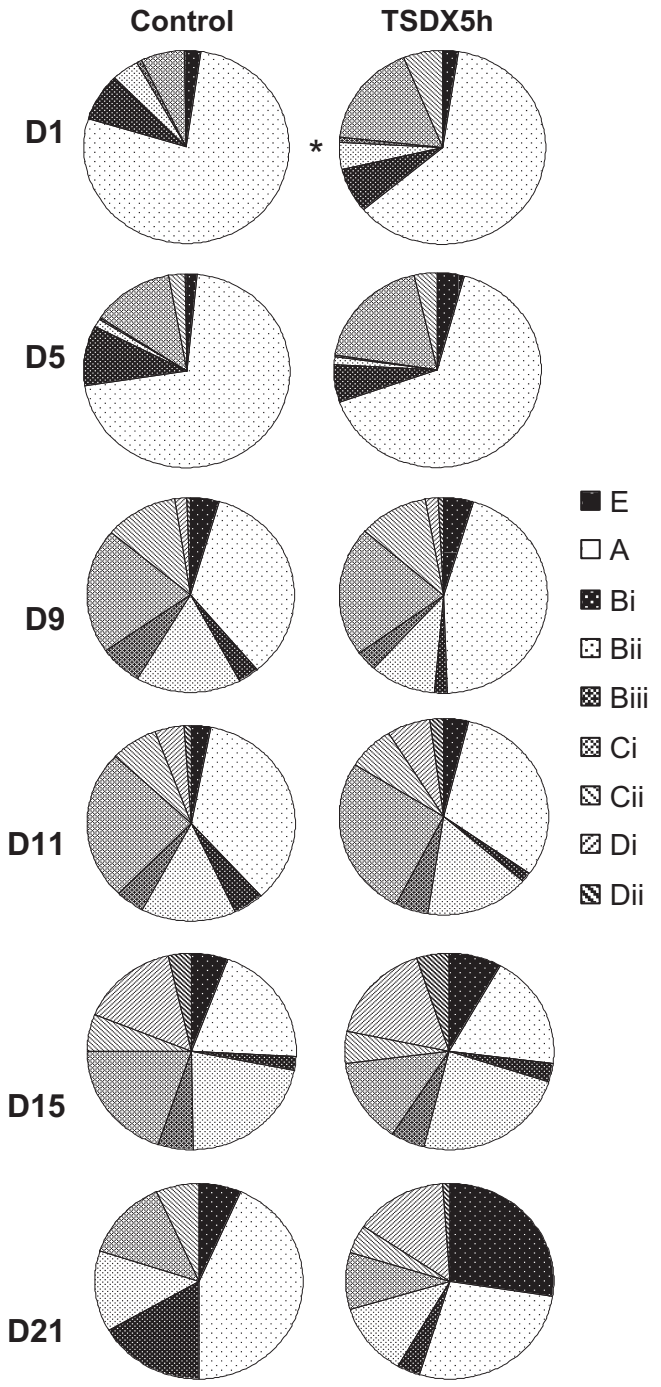

Fig. 3. Distribution pattern of isolation calls during different developmental days in the control and TSDX5h groups. The percent distribution of various calls grouped in different categories obtained on isolation at pnds 1, 5, 9, 11, 15 and 21 are shown in pie diagram for pups in the control and TSDX5h groups. A denotes category consisting of flat calls; B denotes upwards (i), downwards (ii), inverted-U shaped (iii) calls; C is the sum of 2 and 3 syllable calls, D contains wave and complex calls and E stands for dot calls.

TSDX5h group. No differences were observed in the bandwidth of any other call types.

3.6. Amplitude of calls

The majority of calls made by pups in the TSDX5h group were lower in amplitude than those of the pups of the control group. On pnd 1, calls in CF (flat), simple frequency modulation (downward, upwards) and 2-syllable category were significantly less intense than in the control group (Fig. 5). On the subsequent tested day (pnd 5), only MF 2-syllable calls were significantly lower in amplitude than in the control group. On day 9, upwards, inverted U and 2-syllable calls made by the pups of TSDX5h mothers remained significantly lower in amplitude (−34.8 to −48.6; −30 to −45.2; −33.8 to −48.9) than the respective calls of control pups (−28.6 to −40.6; −28.3 to −40.6; −28 to −45.2).

3.7. USV distribution over 1st min

The number of calls made during the initial first minute was higher till pnd 15 in control groups of pups. Similar trends were observed in pups from TSDX5h group. However, calling rate in the TSDX5h group remained slightly higher in comparison to control values during pnds 1 and 5.

3.8. Body weights in pups

The pups born to TSDX5h group were initially having slightly lower body weight than the control pups ($p = 0.025$), but thereafter no statistically significant difference was found between the two groups on all pnds (Fig. 6).

3.9. Anxiety testing in weanling using elevated plus maze

After weaning, the early adolescents were tested in the EPM, during pnds 26–28, to assess their anxiety-related behavior. The time spent in central zone by the juveniles was similar (24.3 and 24.8%) in both the groups (Fig. 7A). However, the pups from TSDX5h group had spent significantly more time (25.01 ± 4.1%) in the open arm zone, which was nearly double in comparison to the control group (12.56 ± 2.2%).

The TSDX5h group weanlings made higher number of entries (28.25 ± 4.8) in the open arm zone in comparison to control (8.3 ± 1.4), as shown in Fig. 7B. The total distance covered during the test period, total time in mobility, and the numbers of line crossings were also higher in TSDX5h group weanlings (Fig. 7C, D, E). Though there was significant increase in ethological parameter like grooming, no change was observed in the head dipping (Fig. 7F).

4. Discussion

The results showed that the pups born to the rats, sleep-deprived during the third trimester of pregnancy, produced increased USVs during the initial days after birth. There were changes in the different components of USVs during these pnds 1–9. But their USVs were diminished on pnd 11, which is the peak vocalization day for control pups. These weanlings of sleep-deprived mothers also showed reduced risk assessment capability.

The total number of calls in the pups of the control group was low during the initial days but it increased and reached to a peak value on pnd 11. Thereafter, the total number of calls was reduced, reaching to a very low value on day 21. The temporal profile of USVs produced by the control group of pups followed a pattern which is comparable to earlier reports [6,12,29]. The pups of sleep-deprived dams made increased USVs during the initial pnds 1–9. As USVs are isolation induced distress calls, it can be assumed that the pups of sleep-deprived dams showed higher sensitivity or responsiveness to maternal isolation stress. It is reasonable to suggest that the enhanced vocalization is a non-specific response to maternal stress, as enhanced USVs were observed in pups born to mothers subjected to acute prenatal stress [30]. If we argue that the changes in USVs are the result of sleep deprivation stress, we should expect increased vocalization in pups after TSD and REM sleep deprivation of dams. As we do not find similar pattern of changes, we have to assume that TSD of dams produce effects that are sleep specific, though it may have similarity to that induced by non-specific stress. The differences in neural circuits involved in NREM and

Table 1
Mean durations of individual call types in different categories between control and TSDX5h groups on various developmental days.

Call types	Day 1		Day 5		Day 9		Day 11		Day 15		Day 21	
	Control	TSDX5h	Control	TSDX5h	Control	TSDX5h	Control	TSDX5h	Control	TSDX5h	Control	TSDX5h
A	44 (19, 80)	49 (24, 128)	51 (21, 77)	55 (30, 85)	28 (11, 70)	51 (9, 82)	41 (15, 110)	49 (6, 92)	36 (15, 148)	55* (30, 85)	10 (10, 37)	18 (8, 39)
Bi	51 (19, 77)	20* (6, 119)	31 (5, 52)	16 (7, 112)	16 (8, 23)	19 (11, 35)	17 (8, 21)	18 (12, 32)	21 (6, 28)	16* (9, 22)	10 (9, 11)	10 (8, 27)
Bii	40 (22, 59)	45 (14, 106)	41 (27, 59)	33 (10, 102)	24 (8, 46)	29 (6, 84)	20 (7, 70)	18 (8, 40)	14 (6, 71)	19 (10, 46)	15 (8, 21)	10 (8, 12)
Biii	21 (13, 29)	26 (13, 90)	24 (22, 26)	9 (7, 22)	19 (10, 27)	18 (6, 29)	18 (10, 68)	15 (6, 28)	15 (8, 72)	14 (7, 38)	NF	NF
Ci	84 (12, 103)	82 (32, 126)	67 (38, 87)	78* (44, 119)	50 (31, 69)	54 (33, 98)	73 (18, 118)	59 (39, 121)	35 (25, 181)	48 (22, 68)	27 (17, 61)	49 (10, 204)
Cii	72 (72, 72)	119 (88, 146)	75 (41, 100)	97 (78, 179)	52 (6, 66)	54 (30, 133)	73 (42, 94)	63 (30, 116)	41 (29, 65)	46 (20, 88)	26 (23, 30)	58 (24, 29)
Di	NF	NF	64 (55, 73)	NF	51 (36, 71)	57 (20, 91)	61 (45, 88)	49 (24, 81)	50 (31, 73)	51 (31, 73)	NF	39 (29, 50)
Dii	NF	NF	56 (56, 56)	NF	84 (52, 100)	70 (44, 101)	45 (39, 88)	65 (35, 82)	69 (58, 94)	79 (39, 120)	NF	50 (50, 50)

The values are presented in ms and are tabulated as median (minimum, maximum) in bracket. NF stands for calls that were 'not found'. A denotes category consisting of flat calls; B having upwards (i), downwards (ii), and inverted-U shaped (iii) calls; C are (i) 2 syllable and (ii) 3 syllable calls and D contains wave (i) and complex calls (ii). The significant changes on comparison of calls between control and TSDX5h are depicted in '*'. The level of significance is $p < 0.05$.

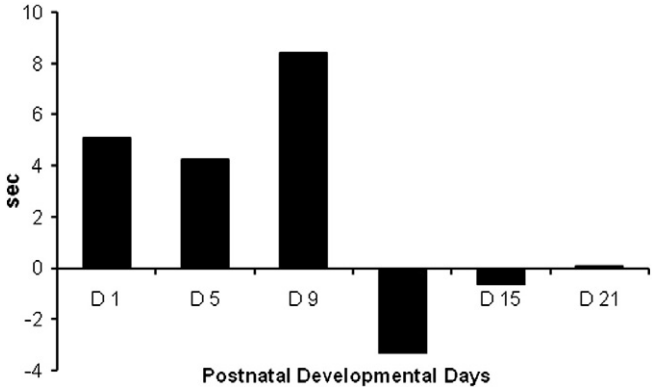

Fig. 4. Plot of excess time spent in ultrasonic vocalization by the pups in sleep challenged mothers. Plot of excess time spent in ultrasonic vocalization by the pups of sleep challenged mothers (difference taken from total time spent in vocalization by control pups) during testing days in the isolation paradigm.

REM sleep may be responsible for non-similarity in TSD and REM sleep deprivation induced changes in USVs.

Diminished USVs in the experimental pups on day 11, which otherwise is referred to as peak vocalization day in control pups, could be described as a dramatic change in the emotional profile of the pups of sleep-deprived mothers. These distress-induced vocalizations emitted by neonates, are the acoustic signals for their mothers to carry out an appropriate retrieval response [12,31–33]. The enhanced USVs during the initial days would have produced increased maternal care, necessitating reduced vocalization of the pups by pnd 11. There are reports to indicate that persistent endearing maternal behavior towards the distressed pups can shape the vocalization response during later development [11,34].

Qualitative changes in USVs were evident in a few parameters like amplitude, durations and peak frequencies. Normally, predominance of flat calls during the initial days indicates that the pups were unable to make frequency modulated calls. The ability to produce frequency modulated calls gradually increased during the later days of growth. However, the pups from sleep-deprived mothers made upward calls of higher pitch (increased bandwidth). In addition, they made longer and higher pitch calls (sharper) in 2-syllable modulated frequency on pnd 5. It is possible that the 2-syllable frequency modulated call of longer duration might be specific and crucial in seeking their mothers' attention. Thus the specific patterns of USVs may be mirroring the alteration in emotional state in rat pups [28]. Such a possibility is strengthened by another report where malnutrition during the prenatal period produced abnormal USVs in pups without altering the calling rates [29]. Even though pups born to sleep-deprived dams were low in

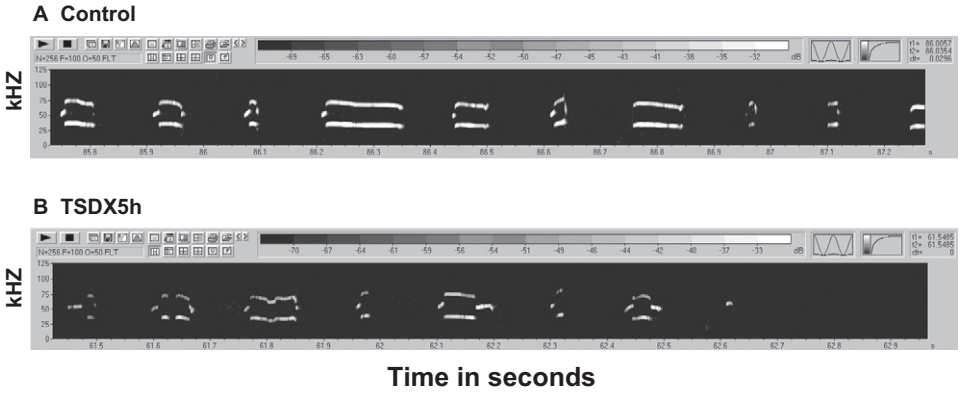

Fig. 5. Representative intensities in ultrasonic calls made by pups in control and sleep challenged mothers. Sonograph of the ultrasonic calls showing alteration in intensities (depicted in gray scale coding of increasing intensity from white to black) obtained in control and the TSDX5h group pups on day 9.

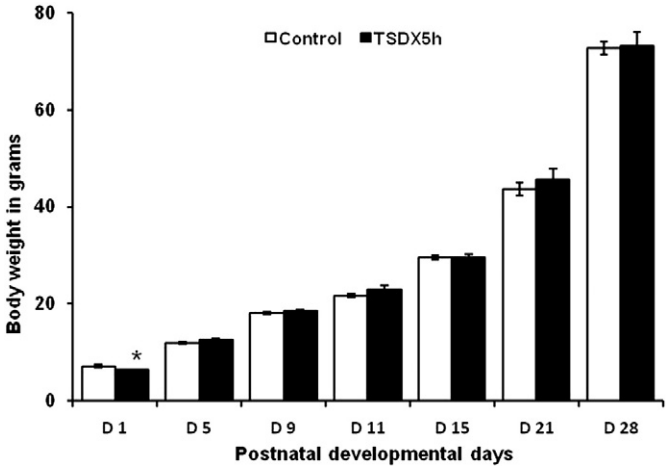

Fig. 6. Body weights of pups on different postnatal days in control and TSDX5h group rats. Body weights of pups in grams are shown in Y axis over different developmental days (X axis) in control and the TSDX5h group. D denotes days and * designates significance value at $p < 0.05$ on comparison of values between control group v/s TSDX5h group.

body weight, they soon recovered their body weight. Recovery of their body weight suggests that the increased USVs during the initial days produced better nursing and care by their mothers.

The findings of EPM test on the weanlings suggest the possibility that the maternal sleep deprivation during prenatal period might manifest in emotional instability and deficits in pups during postnatal life. Increase in the time spent in the open arm and exploratory activities, by the weanlings of sleep-deprived mothers, is indicative of enhanced novelty seeking and risk taking behavior. These results suggest a change in their emotional profile during early adolescent period [35,36].

Though the brain development during postnatal period is more important in determining cognitive function, the impaired neural development during fetal life may increase the chances for deranged emotional development [37,38]. Immature serotonergic systems during pre- and peri-adolescence period make it a critical window vulnerable to various insults even during early development [39,40]. The early manipulations of NMDA receptors in neonates are also reported to affect some components of anxiety during adolescence [41]. The results of the current research support the recent human study associating maternal stress with adverse neural developmental in children [38,42].

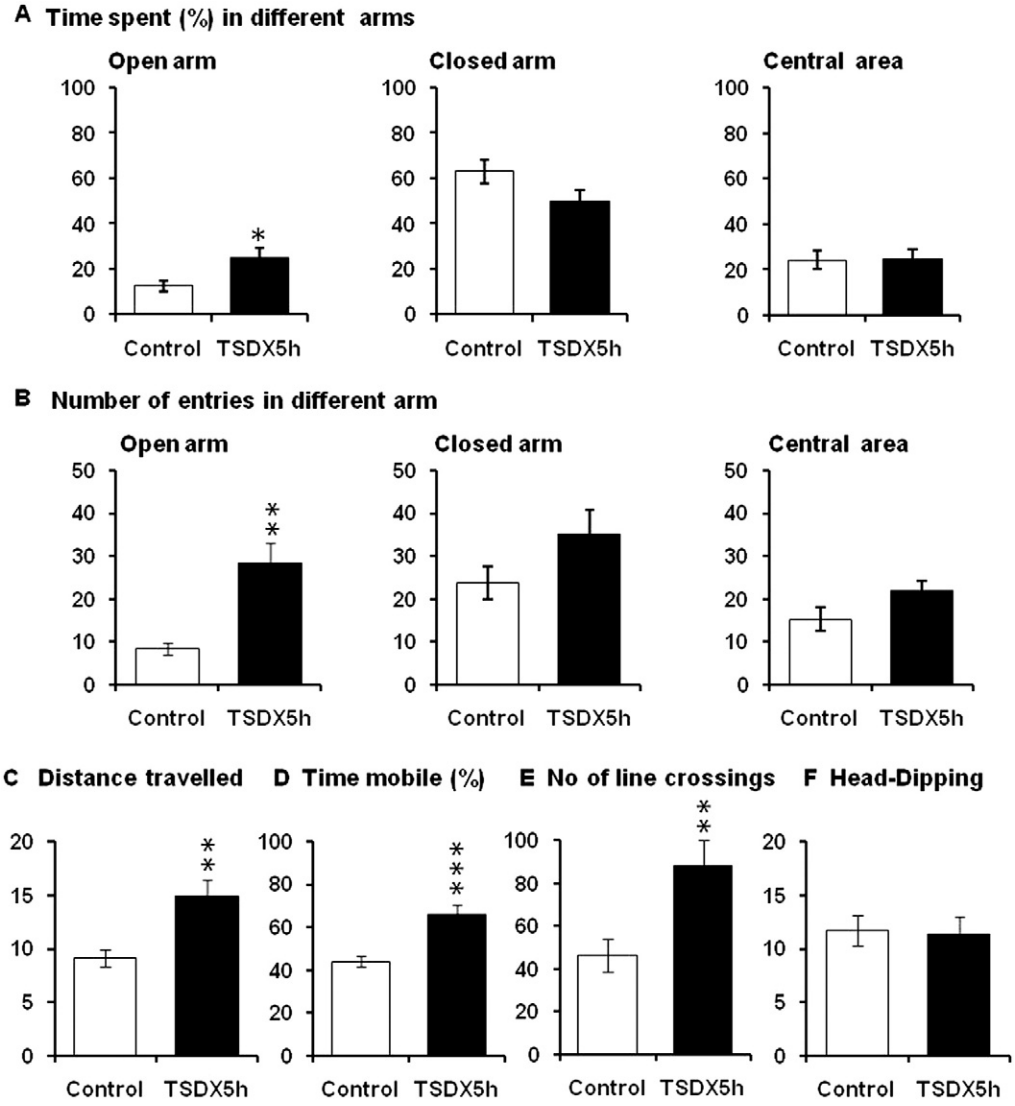

Fig. 7. The comparison of different parameters in control and TSDX5h group rats the EPM test during pnd 25–28. In EPM test, percent time spent in open, closed arm and central area are displayed in A and numbers of entries are shown in B. The open bar represents control and the closed bars are for the TSDX5h group. Percent values are shown in the Y axis in A and D, and numbers in B, E and F. Distance traveled in meters is shown in Y axis in C. Time mobile, number of line crossing and head dipping are shown in D, E and F respectively. * designates significance values $p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

The new-born pups of sleep-deprived dams had slightly lower body weight, but thereafter no statistically significant difference was found between the two groups. Maternal prenatal stress has been found to be significantly associated with low infant birth weight [43]. A previous study had also reported low birth weight in babies of REM sleep-deprived dams [12]. Some of the clinical observations also show that sleep loss during pregnancy leads to fetal growth restriction [12,44–46]. Hormones associated with stress that can pass through the placenta could be responsible for reduced birth weight in prenatally stressed infants [47].

Even in the absence of any overt changes in body weight in early postnatal days, USVs supplemented by behavioral testing during later development may be useful in recognition of anxiety traits in the rodent model. An altered crying pattern with abnormal cries is observed in human babies with brain damage [2]. Since evaluation and assessment of newborn's behavior is comparatively difficult, it is emphasized that the USV measurement in neonates can be taken as a possible tool to study emotional development in rodents [12,48,49].

It must be mentioned here that in contrast to pups, the USVs emitted by adult rodents display more distinct acoustic features and are largely grouped into two broad categories. The long 22-kHz USVs are emitted in adverse conditions such as exposure to painful stimulus, defeat in aggressive encounter, and after ejaculation during mating. On the other hand, short 50-kHz calls are emitted during copulatory act, play with mates and positive motivational states [50–53].

Further studies are certainly required to find out whether the excessive exploratory activities and risk taking behavior are continued during adolescence and adulthood. Though reports of persistent endearing maternal behavior towards the distressed pups [11,34] can explain the blunted response on pnd 11, the role of the optimal and extra-optimal maternal care in the emotional development of the offspring needs further investigation. As we know that the USVs can shape the mother's responsiveness towards the pups [33], it would be interesting to understand further the importance of these acoustic signals. This is the lead report showing an association between maternal sleep loss and increased USVs in neonates and ensuing emotional sculpturing during later development.

Acknowledgments

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